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Original Research Article

Radiologic, Ultrasonic and pathological assessments of locally applied estrogen on promotion of experimentally induced tibial fracture healing in rats

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ABSTRACT

The objective of the present study was to evaluate the ability of estrogen to promote induced tibial fracture healing in a rat model. The study was conducted on 14 albino rats divided into two equal groups (seven rats in each group). The first group considered as a control group. The second group was injected estradiol solution 0.1 mg/kg into the fracture gap. The progress of healing in each group was evaluated by clinical, radiography, ultrasonography and pathological examinations. The healing process was observed to be superior in the estrogen group compared to the control one. Estrogen was found to promote healing of injured bone and is suggested to be used in cases of complicated or delayed bone fracture.

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1. Introduction

Bone has a unique ability to heal and regeneration without forming scar tissue therefore; the main goal of treating fractured bone is to reconstitute bone ends at the site of injury to restore native bone function. However, poor biological or mechanical conditions lead to delayed fracture healing, mal-union, or nonunion (Roth et al., 2017).

Estrogen deficiency results in microarchitectural alterations of trabecular bone in both the mandible and the tibia within 16 weeks (Yang et al., 2003). While, long-term estrogen deficiency in ovariectomized rats decreases the mandibular cortical thickness (Yang et al., 2005a). Estrogen improves fracture mending in osteoporotic bone after osteotomy in rats with unblemished ovaries (Kolios et al., 2009 and Kolios et al., 2010). Estradiol can be delivered both systemically or locally to the bone with differences in concentrations and side effects (Tahami et al., 2016). The application of estrogens enhances fracture healing of long bones in all stages (Beil et al., 2010).

Beil et al., (2011) suggested a role for estrogen in the early inductive phase of endochondral ossification in fracture healing in animals, as it was proved in New Zealand rabbits.

Despite developments of advanced imaging techniques to quantitatively and qualitatively assess bone fracture healing; plain radiography remains the most commonly used radiographic tool for this purpose (Davis et al., 2004).

Ultrasound is able to detect callus formation before radiographic changes are visible (Craig et al., 1999a). The bony surface reflects approximately 56% of incident acoustic waves, while the remaining 44% is absorbed. Therefore structures below the bone surface will not be visualized (Risselada et al., 2003 and Gielen, 2014). However, the changes of the fracture callus (fracture hematoma, formation of a distinct callus, and mineralization of the callus) can be identified by ultrasonography examination (Risselada et al., 2005 and Pozzi et al., 2012).

2. Materials and Methods

The present study was enrolled on 14 healthy male albino rats having a weight 172.71 ± 4.32 gm randomly allocated into two groups (control and estrogen), seven rats in each and housed in cages with solid floors covered with 3cm of soft bedding (hay) and were fed and watered ad libitum. Rats were kept in standard laboratory conditions 22±3°C, 60±5% humidity and a 12 hours light/dark cycle. Animal handling was carried out in accordance with and approved by the institutional animal care and use committee, Faculty of Veterinary Medicine, Beni-Suef University.

Each rat was injected 100 mg/kg ketamine (2 ml) (Ketamine® 5% sol. Sigma-Aldrich Co.); plus 10/kg mg xylazine hydrochloride (2 ml) (Xyla- ject® ADWIA Co. A.R.E) 0.7 ml of the mixture was injected intra-peritoneal by using syringe 23-25 gauge 5/8 inch needle (Flecknell, 2015 and Hohlbaum et al., 2018).

Tibiae fracture induced by bending the lower part against an artery forceps which applied externally, the fractures were confirmed radiographically according to (Handool et al. 2018) and external fixation retained by casting tape.

The first group was left as control and in the second group estrogen; tibiae was locally injected by 17α-ethynyl estradiol, 0.1 mg/kg (Estradiol valerate 100mg/ml (Estradiol valerate® PERRIGO) at the side of fracture according to (Kolios et al., 2010). (Fig. 1 and Fig.2).

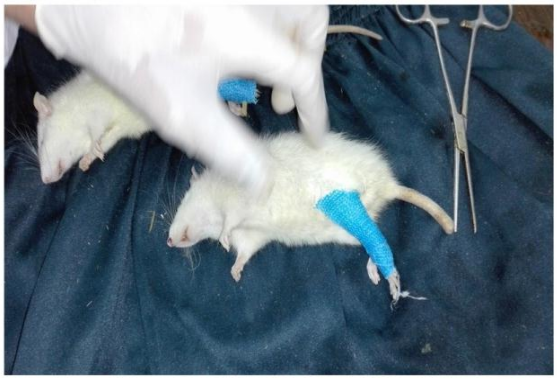


Fig.1 Photograph showing estrogen injection at the induced fracture site of rat tibia.	Fig. 2 Photograph showing cast application after estrogen injection.

For radiological evaluation, Tibiae lateral views were taken by the Faxitron Cabinet X-ray System **(**Hewlett-Packard, 50 µm x-ray beam output; model 43855A; IL 60089, USA). High-resolution 30x40 cm films (Fuji HR-E 30 Medical X-ray) were used and radiations at 48 kVp and 15 mAs, at a 70 cm focal film distance (F.F.D.) were done. The description and evaluation of the fracture healing (fracture gap and callus formation) was performed for all rats according to (Risselada et al., 2005).

Diagnostic ultra-sonographic machine (Mindray dp 10 Germany vet with multiple elements convex probe and a 7.5 MHz frequency), used to follow up the healing process with the aid of coupling gel to avoid air buckets after removal of casting tape (Risselada et al., 2005).

The shafts of tibiae were harvested from rats, removing the surrounding attached musculature, the bone specimens were then fixed in 10 % neutral buffered formalin for 48 hours. After fixation, bone were decalcified using a buffer solution of 17 % EDTA disodium solution (Ethylenediamine tetra-Acetic acid disodium salt B.P.93®: El Nasr pharmaceutical chemical, Egypt) for one month during this period, all specimens were weekly inspected for signs of complete decalcification (Shibata et al., 2000)**.**

After complete decalcification, the bone specimens were washed in running tap water for 12 hours, then dehydrated in ascending grades of ethyl alcohol (70 %, 80 %, 90 % and 96 %) (Absolute 1, Absolute 2 and Absolute 3), then cleared by using xylene (xylene I, xylene II and xylene III) and finally, the bone specimens embedded in soft paraffin (paraffin 1, paraffin 2 and paraffin 3) then blocked in hard paraffin wax, sectioned 5-7 µ and stained with routinely Hematoxylin and Eosin according **to** (Bancroft, and Marilyn, 2008 and Fathy et al., 2017).

3. Results

Results of First week post fracture induction

By the end of first week, Radiography of tibiae of control group; fracture line was clearly visible, the gap was clearly visible and radiolucent (Fig. 3), while in estrogen group fracture line was less visible with clear evidence of callus formation; the gap was less visible Fig. 4). Ultrasonography of control group (Fig. 5), showed that the bone surface was irregular in outline. Hypo-echoic regions indicate the presence of a hematoma. And estrogen group (Fig. 6) Showed that the fracture site is filled with tissue with a mixed hypoechogenic and anechogenic appearance. The callus has an inhomogeneous and irregular appearance with areas of hyperechogenicity indicating the start of mineralization.

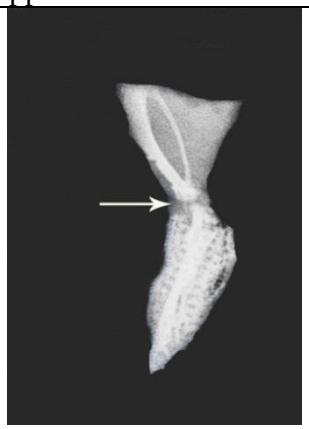


Fig. 3 Radiograph of control group after first week; X-ray shows a transvers fracture of the tibial shaft below the fibular junction fracture edges are clearly visible and radiolucent (arrow).

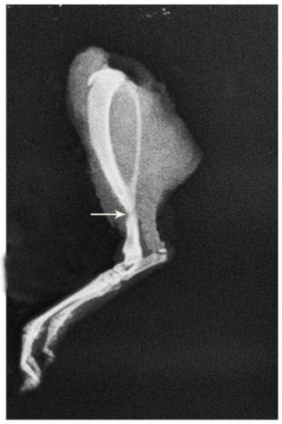


Fig. 4 Radiograph of estrogen group after first week; X-ray shows a transvers fracture of the tibial shaft below the fibular junction fracture edges are less visible radiograph shows periosteal new bone separated from the underlying cortex by thin radiolucent line (arrow).

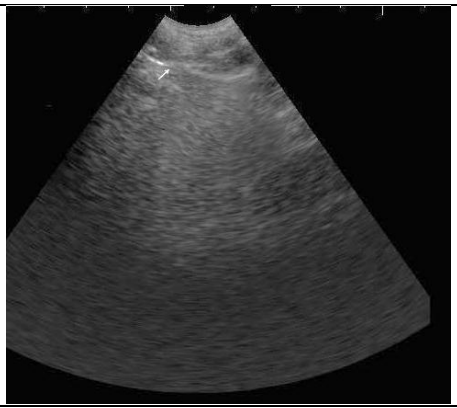


Fig. 5 Ultrasonogram of control group after first week showing that the bone surface was irregular in outline. Hypo-echoic regions indicate the presence of a hematoma.

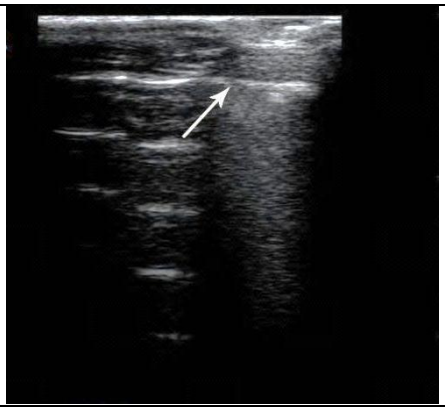
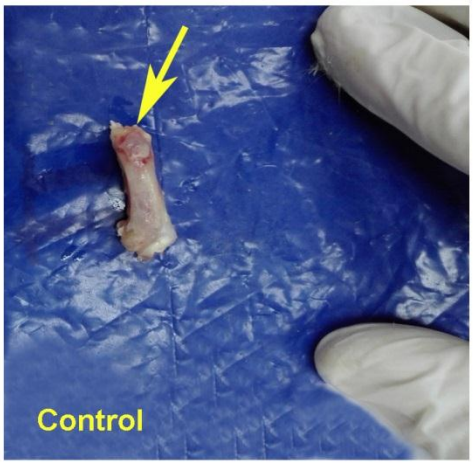


Fig. 6 Ultrasonogram of estrogen group after first week showing that the fracture site (arrow) is filled with tissue with a mixed hypoechogenic and anechogenic appearance. The callus has an inhomogeneous and irregular appearance with areas of hyperechogenicity indicating the start of mineralization.

By gross pathological examination of the induced fractured site in the tibial shaft after one week for both control and estrogen injected group; the control group showed red wide inflammatory area around the fracture site Fig. (7), while in estrogen injected group, this inflammatory area disappears Fig. (8).

By the histopathological examination, the control group showing the end of the inflammatory phase as remnant granulation tissue was filling the fracture gap; which was rich by blood capillaries, and entangled lymphocytes, while bone fragments are still present in the site of the induced fracture Fig. (9), the estrogen injected groupshowing remnant of small blood capillaries of the granulation tissue associated with active proliferating fibrocytes filling the fractured gap Fig. (10).



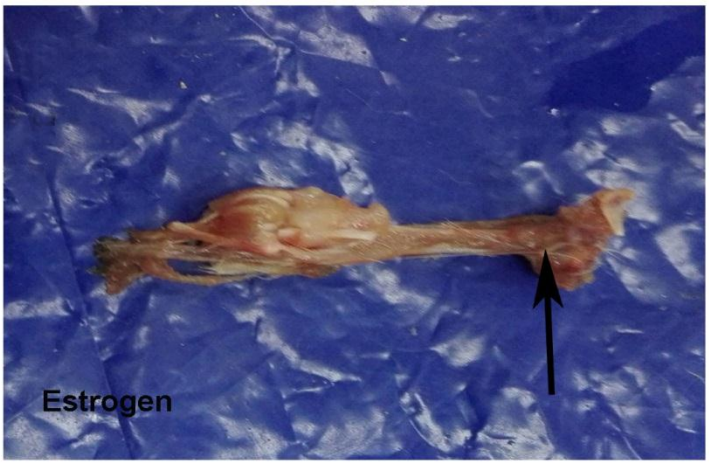
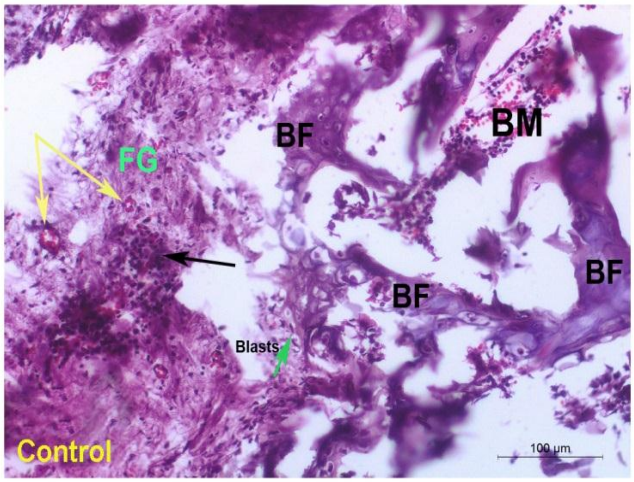


Fig. 7 Photograph of rat tibia of control group after being harvested from the rat post-fracture by one week showing inflammatory red zone at fracture site (yellow arrow).	Fig. 8 Photograph of tibia shaft after being harvested from the rat of estrogen injected group post-fracture by one week showing minimal inflammatory area at the fracture site (black arrow).



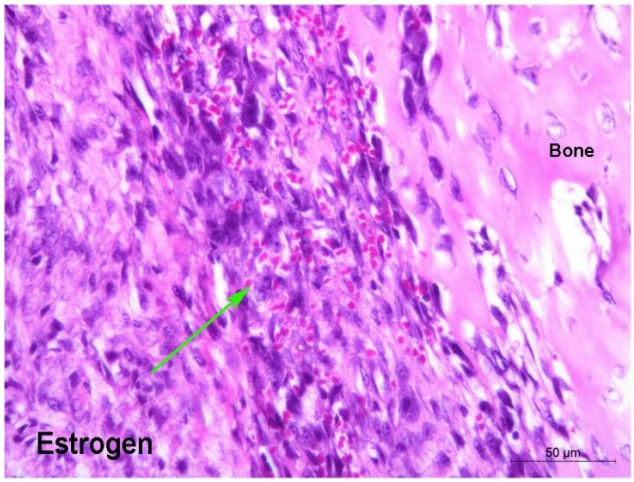


Fig. 9 Photomicrograph of rat tibia of control group post fracture by one week showing granulation tissue filling fracture gap (FG), rich by blood capillaries (two yellow arrows), and entangling in it lymphocytes representing the end of the inflammatory phase of the fracture (black arrow), while bone fragment (BF) Bone marrow (BM) (H&E; Bar= 100 µm).	Fig. (10): Photomicrograph of rat tibia of estrogen injected group post-fracture by one week showing remnant of small blood capillaries associated with active proliferating fibrocytes filling the fractured gap (green arrow) (H&E; Bar= 50 µm).

Results of Second week post fracture induction

In the second week, on radiography, revealed the appearance of peripheral callus and little bone bridges, whereas the gap between fracture edges is still visible in control group (Fig. 11), while in estrogen group fracture line start to disappear and callus formation was visible, fracture gap tends to be radiopaque. Meanwhile, on ultrasonography a global formation is evident related to the periosteal collars that tend to meet from the two sides of the fracture filling the gap. Hyper-echoic lines of tibia; in-betweens (fractured area) appeared as gray pitched hypo-echoic small gape denotes the start of callus formation in control group (Fig. 11) and estrogen group with more extent (Fig. 12).

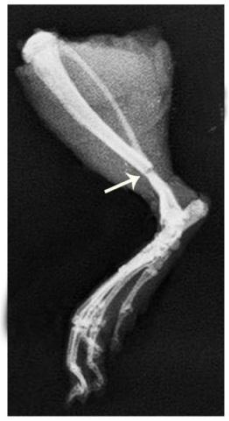


Fig. 11 Radiograph of control group after second week; fracture edges is still visible as the X-ray shows a transvers fracture of the tibial shaft below the fibular junction.

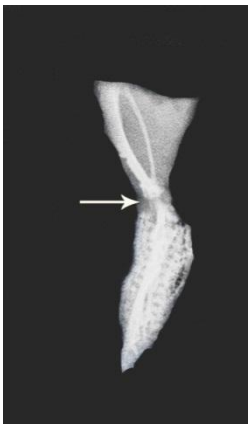


Fig. 12 Radiograph of estrogen group after second week; X-ray shows a transvers fracture of the tibial shaft below the fibular junction showed beginning of callus formation & bridging of gap.

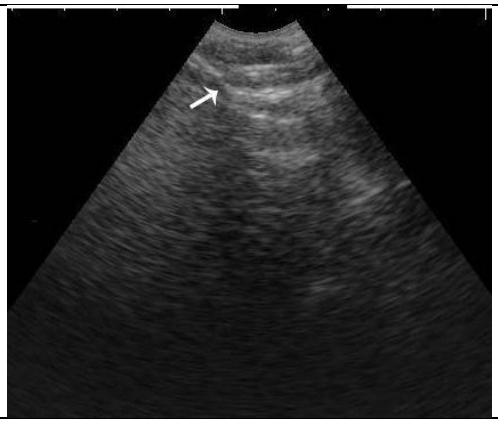


Fig. 13 Ultrasonogram of control group after second week showed hyper-echoic lines denote fractured bones in-between gray pitched hypo-echoic small gape denotes start of callus formation

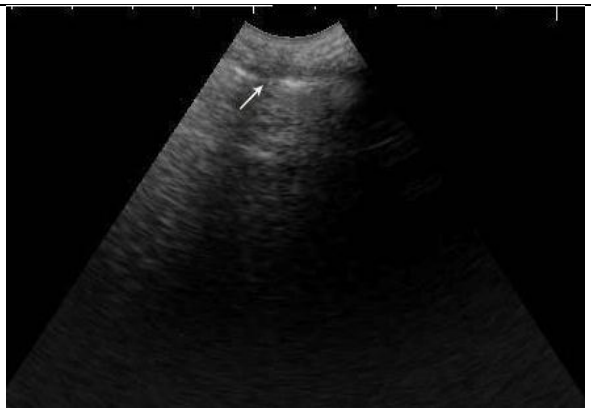


Fig. 14 Ultrasonogram of estrogen group after second week showed that presence of reflective, irregular interfaces within the osteotomy gap indicative of bone production

Fig. 15 Photograph of rat tibia of control group post fracture by two weeks showing beginning of formation of hard callus at fracture site (yellow arrow).

Fig. 16 Photograph of rat tibia of estrogen injected group post fracture by two weeks showing formation of hard callous at fracture site (black arrow).

By gross pathological examination of the induced fractured site in the tibia shaft after two weeks for both control and estrogen injected group; the two groups showed hard callus formation Fig. (15) and Fig. (16).

By histopathological examination, the control group showed overfilling of the fractured gap with active fibrocytes, associated with active oesteoblasts as main structure of hard callus while all area of the fracture was clear from any erythrocytes and inflammatory cells Fig. (17). In the estrogen injected group, there was narrowing and short distance between the two bony edges in which there is an active oesteoblasts associated with fibrocytes Fig. (18).

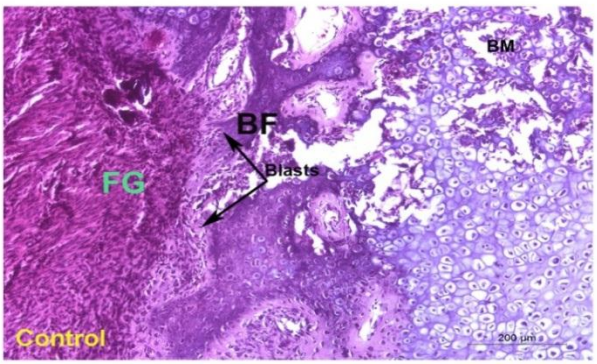


Fig. 17 Photomicrograph of rat tibia of control group post fracture by two weeks showing overfilling of the fractured gap by active fibrocytes, associated with active oesteoblasts (two black arrow) (H&E; Bar= 200 µm).

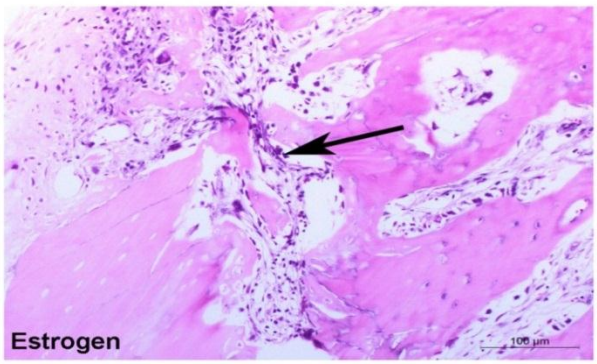


Fig. 18 Photomicrograph of rat tibia of estrogen injected group post fracture by two weeks showing narrow distance between the two bone fracture edges, associated with active oesteoblasts and minimal fibrocytes (black arrow) (H&E; Bar= 100 µm).

Results of Fourth week post fracture induction

In the fourth week, Radiography revealed the bone trabeculae extend from one fragment to the other, the solution of continuity dissolves, and the callus formation is completed. Fracture line is barely visible; callus bridges the fracture edges and the area in between become radiopaque Fig. 19). in control group, while in estrogen group callus is clearly visible and condensed with incomplete disappearance of fracture line Fig. 20), Meanwhile, on ultrasonography, the echo reflected by the focus increases in intensity according to the initial callus calcification; the collars meet in one hyper-echogenic convex, bridge-shaped structure on the fracture gap.

The hyper-echogenic structure represents a clear obstacle to the ultrasounds, and an acoustic shadow appears below the newly formed periosteal callus according to its progressive calcification. Hyper-echoic lines tibia; at the fractured area, appeared as pitched hyper echoic denotes incomplete callus bridging in control group (Fig. 21) and in estrogen group, the fractured area appeared as hyper-echoic area revealed condense callus formation; hypo-echoic fracture line nearly invisible (Fig.(22).

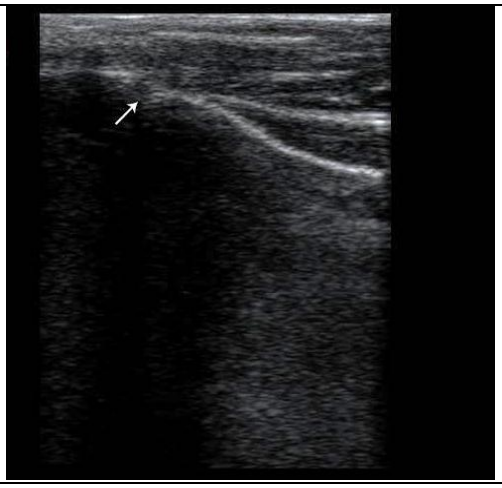


Fig. 21 Ultrasonogram of control group after fourth week, Hyper-echoic lines denote fractured bones; in-betweens pitched hyper echoic denotes incomplete callus bridging.

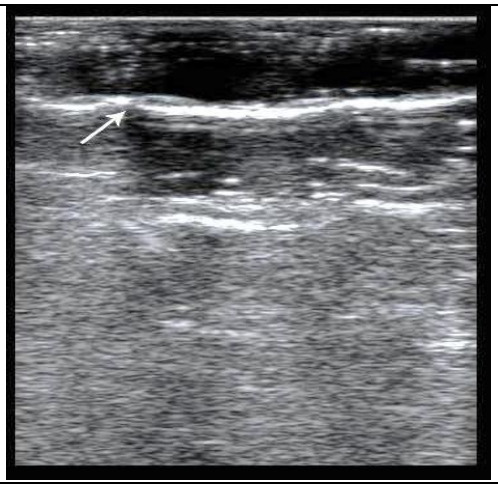


Fig. 22 Ultrasonogram of estrogen group after fourth week. The bony surface is continuous and hyperechoic.

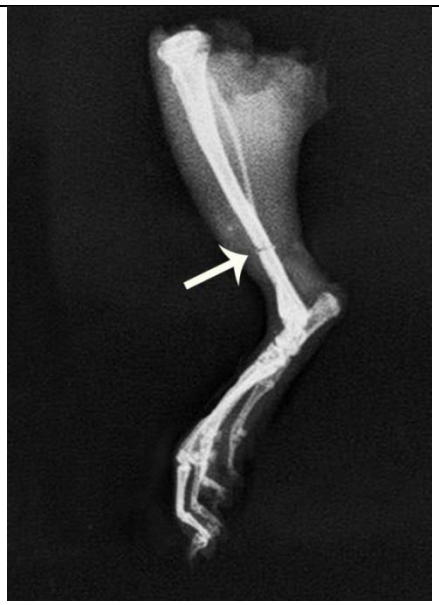


Fig. 19 Radiograph of control group; X-ray shows a transvers fracture of the tibial shaft below the fibular junction after fourth week remodeling not complete.

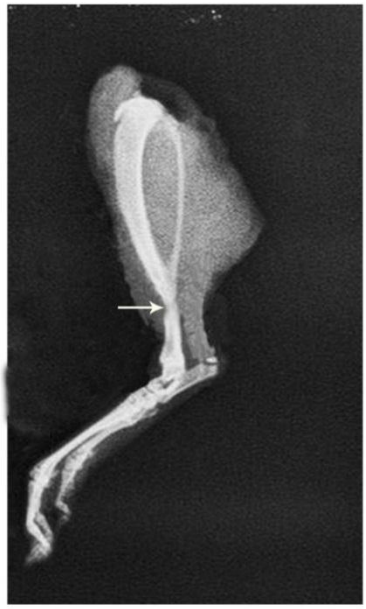


Fig. 20 Radiograph of estrogen group; X-ray shows ill prominent transvers fracture line of the tibial shaft below the fibular junction after fourth week. The callus is clearly visible and condensed.

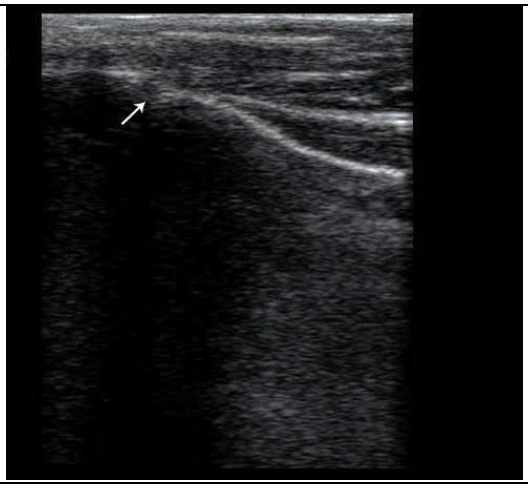


Fig. 21 Ultrasonogram of control group after fourth week, Hyper-echoic lines denote fractured bones; in-betweens pitched hyper echoic denotes incomplete callus bridging.

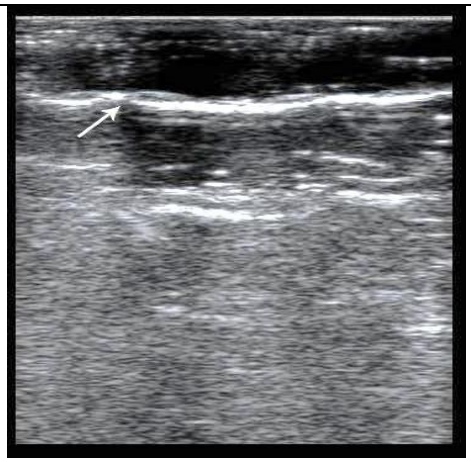
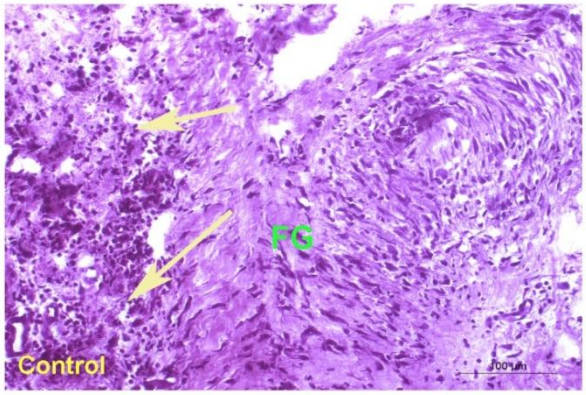


Fig. 22 Ultrasonogram of estrogen group after fourth week. The bony surface is continuous and hyperechoic.

By histopathological examination of the induced fractured site in the tibiae shaft after four weeks for both control and estrogen injected group; the control group showed an area of organization represented by fibrocytes associated with an active proliferation of fibers filling the fracture gap represent hard callus phase of bone healing Fig. (23), while in the estrogen injected group, the site of the fracture showed active oesteoblasts for beginning of the

remodeling phase with minimal fibrocytes remained from the previous hard callus formation phase Fig. (24).



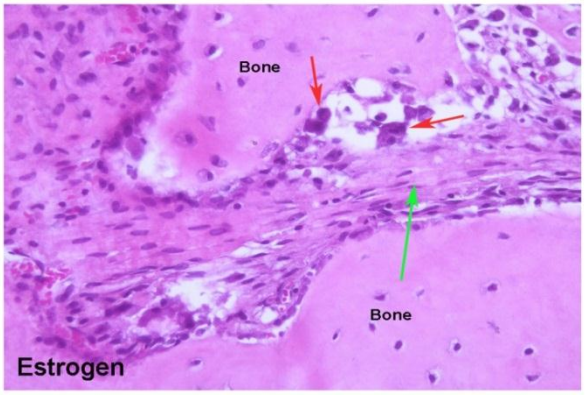


Fig. 23 Photomicrograph of rat tibia of control group post fracture by four weeks showing the organization of fibrocytes (yellow arrows) associated with an active proliferation of fibers filling fractured gap (green), (H&E; Bar= 100 µm).	Fig. 24 Photomicrograph of rat tibia of estrogen injected group post fracture by four weeks showing fractured gap area (green arrow), while osteoclasts were seen in the area for remodeling (red arrows), (H&E; Bar= 50 µm).

4. Discussion

Results of the current study supported the hypothesis assumed that the use of a closed fracture model is considered the choice model for studying the fracture healing process. This is important because the periosteum and the soft tissue surrounding the fracture site play an important role during the fracture healing processes (Tatari et al., 2007b, Davis et al., 2015 and Haffner-Luntzer et al., 2016) in our study; the closed tibial fracture model in rat has been chosen.

In our study, high effectiveness of ultrasound examination in evaluation of bone healing was proved, which was in consequence with (Moed et al., 1995, Moed et al., 1998a, Craig et al., 1999a and Griffith et al. 1999). As there were many advantages of ultrasonography more than X- rays such as no ionizing radiation, qualitative evaluation of the callus formation, painless and well tolerated.

Female sex hormones appear to be compulsory, not only for the conquest of peak bone mass in both sex, but also for the keeping of bone mass (Ahlborg et al., 2003, He et al., 2012 and Tahami et al., 2016).

This study was done to evaluate the local effect of exogenous estrogen on promoting fracture healing in adult male rats. Few studies were evaluated the effect of estrogen treatment on fracture healing by doing ovariectomy and evaluated the effect of estrogen on bone repairing in the absence of endogenous estrogen (Negulesco and Eglitis, 1975, Yang et al., 2003 and Beil et al., 2011). So we replaced the female overioectomizied rats by adult male rats to evaluate the effect of exogenous estrogen.

Radiographic evaluation revealed that the union rate is higher in estrogen treated groups comparing with control one. These findings were agreed with (Miwa et al., 1999, Bolander, 1992, Namkung-Matthai et al., 2001, Cao et al.,

2002 and Beil et al. 2011) who also added that, the union and reduction of fracture gap rates were higher in locally treated group comparing to systemic administration.

Fractured bone healed by regeneration, and this process completed when the fractured bone site pass through four main subsequent phases including; phase of hematoma formation directly on the time of the fracture, (its presence associated with the bony necrotic fragments as well as destructed surrounding tissues as musculature, blood vessels, nerves and lymphatic's) initiate the attraction of inflammatory cells at the fracture site (beginning of the inflammatory phase), after that the fibroblasts and fibrocytes begin to appear (as the reparative phase or callus formation) to narrow the gap between the fracture edges, matured forming collagenous bridge between the fracture edges, (the remodeling phase) begin when the osteoclasts proliferate to remove the excess of bone formed.

It is a good thing that connective tissues of the body have the capacity to heal themselves, because fractures can heal. In the region of the break, new blood vessels grow to form a highway carrying cells that can clean up the mess and can start forming new connective tissue. And these fibrocytes were seen in the site of the fracture in the second group of estrogen after one week post fracture induction in our results which supposed that these fibroblast and fibrocytes can produce collagen in the region of the fracture (a mass of collagenous material forms) and this is called a callus, which stabilizes the fracture and creates a template on which new bone can be laid down. On other hand, the examination of the fracture site of first control group after one week post fracture induction, the fracture gap was still having some bony necrotic fragments as well as some remained erythrocytes and inflammatory cells.

So, by the end of the first week post the fracture, the control group showed the inflammatory phase, while the estrogen injected group showed beginning of the reparative phase. Where the fibroblasts and the fibrocytes started to begin in appearance at the fracture site in the control group by the end of the second week post fracture. The estrogen group showed minimal distance gap between two fracture edges at the fracture site due to contraction of the already formed fibrocytes in the end of first week post the induction of the fracture which shrink the fracture gap.

By examination of two groups in the fourth week, the second group of estrogen showed beginning of the remodeling phase which confirmed by the appearance of bone macrophages (osteoclasts) around the fracture gap as well as a proliferation of osteoblast (the main cells of bone healing or regeneration). While the first group was still having the fibrocytes in between fracture edges (in the reparative phase).

Estrogen local application was found to be a promoter as faster bone healing in long bone of rats, which enhances all stages of bone healing and these results were agreed with (Beil et al., 2011) who proved the same results in New Zealand rabbits.

In this study there was a significant difference between estrogen group and control group in terms of early disappearance of the fracture line and bone density.

5. Conclusion

The second group which is the estrogen injected group was faster with shorter time of different bone healing phases than the control group by the different assessment methods, so the use of estrogen is suggested to be used in cases of complicated or delayed fracture.

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